

# Fluorescent phallotoxin, a tool for the visualization of cellular actin

(microfilaments/rat kangaroo PtK1 cells/bovine kidney MDBK cells)

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**ABSTRACT** A fluorescent derivative of phalloidin has been synthesized possessing high affinity to filamentous actin. This compound was used for visualization of actin-containing structures in eukaryotic nonmuscle cells. Due to its low molecular weight (1250), fixation by formaldehyde was sufficient to render the membrane permeable for the labeled peptide. Bundles of microfilaments are the predominant pattern in the flat rat kangaroo PtK1 cells, whereas a net of concentric fibers characterizes the more spherical bovine kidney MDBK cells. Specificity of staining was confirmed by competition experiments with unlabeled phalloidin.

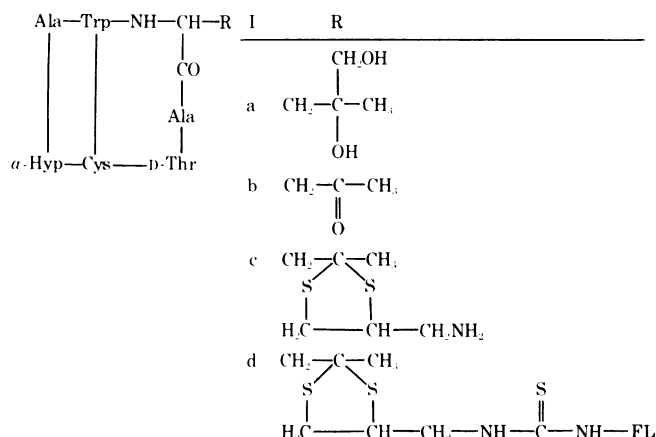
The phallotoxins, a family of poisonous bicyclic heptapeptides from the mushroom *Amanita phalloides* (for review see ref. 1), whose main representative is phalloidin (Ia), form tight complexes with F actin from liver cells and from muscle. As a consequence of their phalloxin binding, the filaments become strongly stabilized against various chemical and physical stresses like 0.6 M KI, heat, proteolytic enzymes, ultrasonic ruptures, and pH changes (for review see ref. 2). Though some structural features of the phalloidin molecule are indispensable for its toxicological action and its affinity for F actin, the side chain of amino acid 7 ( $\gamma$ - $\delta$ -dihydroxyleucine) is accessible to chemical modifications without significant alterations of the binding capacity. Thus, by several derivatization steps we were able to synthesize a fluorescent phalloidin derivative (Id) (FL is fluorescein).

Eukaryotic mammalian cells also contain contractile proteins such as actin and myosin and changes in cell structure (e.g., cell movement and cytokinesis) can be traced to contractions initiated by these proteins (for review see ref. 3). The cytoskeletal organization of actin that polymerizes into filaments has been demonstrated for various established cell lines, with the help of actin-specific antibodies, by immunofluorescence microscopy (4, 5) and by decoration with fluorescein-labeled heavy meromyosin (6) because microfilaments, but not other intracellular fibers, bind heavy meromyosin (7). In this study fluorescent phalloidin was used to visualize F actin bundles in two mammalian cell lines.

## MATERIALS AND METHODS

Fluorescein isothiocyanate (isomer A) was from Fluka (Buchs, Switzerland). Kieselgel was from Merck (Darmstadt, West Germany). If not otherwise noted, the chromatographic procedures were performed on Sephadex LH-20 in 1.5 × 250 cm glass columns under hydrostatic pressure. Elution was monitored at 280 nm by an LKB Uvicord II optical unit. Binding constants to rabbit muscle F actin were measured by equilibrium dialysis. F actin was a gift of P. Dancker (Department of Physiology, Max-Planck-Institute for Medical Research).

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**Reagent Preparation.** Ketophalloidin (Ib) was prepared from Ia according to Wieland and Schöpf (8). 1-Aminopropane-2,3-dithiol was synthesized from 1-amino-2,3-dibromopropane (9) according to Dix and Bresson (10). Synthesis of Ic was as follows. Ketophalloidin (Ib, 151.2 mg, 0.2 mmol) was dissolved in 1 ml of a solution of methanol saturated with HCl gas at 0°C containing 98.4 mg ( $\approx$ 0.8 mmol) of 1-aminopropane-2,3-dithiol and stirred for 30 min at the same temperature. The compound was purified, after drying under reduced pressure, by chromatography with a mixture of methanol/water/acetic acid, 1000:1000:15 (vol/vol) as the eluant, followed by a second chromatography on a column with H<sub>2</sub>O/2M NH<sub>3</sub>, 1000:2 (vol/vol). Yield of Ic was 115 mg (67%). FL-phalloidin (Id) was prepared from Ic (17.2 mg, 0.02 mmol) in 1 ml of a 0.5 M NaHCO<sub>3</sub> buffer (pH 9.5) in water/methanol, 7:3 (vol/vol) by adding fluorescein isothiocyanate (7.8 mg, 0.02 mmol) and stirring at room temperature for 5 hr. After chromatography with water/methanol, 50:50 (vol/vol) and a final purification on a short (0.7 cm × 60 cm) Kieselgel 60 column with CHCl<sub>3</sub>/methanol/H<sub>2</sub>O 65:25:4 (vol/vol) as the eluant, 13.2 mg of Id (52.8%) was produced.

**Cell Culture.** The rat kangaroo PtK1 cell line was grown in minimal essential medium containing 10% fetal calf serum. Cells of the Maden-Darby bovine kidney MDBK cell line were maintained in modified RPMI 1640 medium with 10% fetal calf serum. Cells to be stained were subcultured on glass coverslips for 24–48 hr before use.

**Staining of Cells.** Cells grown on glass coverslips were rinsed briefly in phosphate-buffered saline (P<sub>i</sub>/NaCl) (pH 7.4), fixed for 5 min in 3.7% formaldehyde in P<sub>i</sub>/NaCl at room temperature and washed extensively in P<sub>i</sub>/NaCl. After fixation, cells were either used directly for fluorescent staining or dehydrated in absolute acetone for 4 min at –20°C and air dried or rendered permeable by a 2-min exposure to 0.1% Triton X-100 in

Abbreviations: FL, fluorescein; P<sub>i</sub>/NaCl, phosphate-buffered saline.

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P<sub>i</sub>/NaCl at room temperature followed by thorough washing in P<sub>i</sub>/NaCl (11). FL-phalloidin was applied to the cells at a concentration of 0.05 mg/ml in P<sub>i</sub>/NaCl containing 1% dimethyl sulfoxide and allowed to react for 40 min at room temperature in a humid atmosphere. Coverslips were washed with several changes of P<sub>i</sub>/NaCl (for at least 30 min) and mounted on microscope slides in a mixture of 30% glycerol and 70% P<sub>i</sub>/NaCl (vol/vol). Cells were examined in a Zeiss photomicroscope III equipped with epifluorescence and phase contrast optics. Photographs were taken with Kodak Tri-X pan film.

As a control for the specificity of the staining reaction, cells were preincubated for 40 min at room temperature with an excess of unlabeled phalloidin (5 mg/ml in P<sub>i</sub>/NaCl) before and after incubation with FL-phalloidin.

## RESULTS

Unfixed rat kangaroo PtK1 or bovine kidney MDBK cells treated with 0.1 mg of FL-phalloidin per ml and incubated for 2 hr at room temperature showed no fluorescent staining either inside the cell or on the outer part of the cell membrane. This agrees with the earlier findings that phalloidin is not able to penetrate the membrane of these unfixed cells in amounts sufficient to cause lesions (1, 12–14). However, when cells were fixed with formaldehyde, filamentous structures, the so-called microfilaments, could be detected as early as 30–40 min after incubation with the fluorescent phalloidin derivative. For both cell lines (PtK1 and MDBK), such decoration could still be detected at FL-phalloidin concentrations as low as 0.002–0.005 mg/ml. FL-phalloidin revealed a fiber system similar to that reported by various authors using other staining methods (4–6). In contrast to the procedures with actin-specific antibodies and indirect immunofluorescence, treatment with acetone or Triton X-100 is not an obligatory step in labeling actin directly by FL-phalloidin (15). When the acetone or Triton X-100 step was omitted, identical staining of the microfilaments was observed.

Actin fiber distribution varied among the two cell lines examined. Although we could assign for each species a predominant staining pattern (typical patterns are shown in Figs. 1A and 3A), each cell within a culture displayed variations in substructures according to its individual shape. The well-spread PtK1 cells exhibited, as a general feature, an intensive staining of straight fibers running parallel to each other throughout the entire length of the cell. Differential focusing revealed that the fiber system occurred only in the plane that was in contact with the substrate on which the cells were growing (4, 5). In confluent cells, these fiber bundles frequently appeared to continue into the neighboring cell (see arrows in Fig. 1A). However, a more intense fluorescence on the fibers in the intercellular space probably indicates junctions of joint fibers. The fibers originate separately at a certain distance from the cell periphery and frequently appeared to branch out or reconverge (Fig. 1B). Furthermore, actin was also found near the cell periphery in a gauze-like structure. As seen in Fig. 1C, the actin distribution here is arranged quite differently from the microfilament bundles in the cell body and consists of thin, often interwoven, threads (16). These structures were frequently seen in pseudoruffles and have been described by Small and Celis (17) in negative-stained electron micrographs of spread 3T3 cells.

In mitotic cells, actin is not organized into bundles; only a uniform diffuse fluorescence is found in the cytoplasm, leaving the chromosomes unstained (18–20). However, these cells also possess numerous, intensely stained retraction fibers radiating from distinct areas of the cell membrane, which by themselves fluoresce brightly (Fig. 2).

Contrary to the flat PtK1 cells, the visualized actin in the more spherical MDBK cells is arranged in a thin, concentric fiber pattern around the nuclear region. These circles are formed by many thin, relatively short fibers surrounding the nucleus and varying in concentration toward the cell periphery. From these circular filaments, other fibers of various length diverge radially like spokes toward the cell cortex and seem to stretch and fix the concentric structures (Fig. 3A). When MDBK cells, probably in other stages of cell spreading, show a more stretched rather than round morphology, they additionally possess long straight bundles spanning the whole length of the cell similar to those observed in PtK1 cells (21). A different staining pattern, often visualized in the pseudopodial protrusions of MDBK cells, is shown in Fig. 3B. Here we found a polygonal array of actin fibers arranged in a complex network with many connecting points (22). An additional characteristic often observed in MDBK cells is bright fluorescence of the ruffled membrane, but without recognizable fine structure (Fig. 3C). We found differences in the staining patterns of the nuclear regions depending on the fixation procedures used. In cells treated with formaldehyde and in cells treated with formaldehyde/acetone no or only a dim fluorescence could be observed in the nuclei, whereas the Triton X-100 extraction resulted in an intense, but structureless, nuclear fluorescence. Likewise, treatment of analogously fixed cells with fluorescein isothiocyanate, inactivated by ethanolamine, led to an equal staining of the nuclei (data not shown). We suggest that this nuclear staining is due to an artifact.

As a control, to show the specificity of the labeled phalloidin, cells were incubated with 5 mg of native unlabeled phalloidin per ml prior to treatment with FL-phalloidin. By this competitive reaction the staining of actin-containing filaments could be completely inhibited (Fig. 4). Similarly, incubation of the cells with phalloidin after staining with FL-phalloidin also led to the disappearance of the specific fluorescence patterns. Staining of the cell nuclei could not be totally removed by competition with unlabeled phalloidin.

## DISCUSSION

Since 1974, when Lazarides and Weber (4) began to investigate the cytoskeleton by an immunofluorescence technique, actin filaments have been visualized in numerous experiments. In all cases proteins of high affinity to actin were used, among them antibodies to actin (4, 5) and the heavy meromyosin fragment (6, 7). Even the moderate affinity to filamentous actin of DNase I proved sufficient to visualize actin fibers in certain cell lines (23).

Phalloidin possesses high affinity to actin filaments and has a low molecular weight (788) (1). The apparent dissociation constant ( $K_d$ ) of this peptide from rabbit muscle actin has been determined to be as low as  $3.6 \times 10^{-8}$  M (24). There is a good deal of evidence that the toxin also binds to nonmuscle actin with a comparable affinity (25). The high affinity to actin was nearly unchanged after derivatization of the toxin at the side chain of dihydroxyleucine, even after an aminomethyldithiolano group was introduced. Only upon further derivatization by introduction of the bulky fluoresceino group was the affinity reduced to 0.13 that of phalloidin. However, the remaining affinity ( $K_d$  about  $2.7 \times 10^{-7}$  M) can be expected to be high enough to still permit a specific labeling of actin filaments in cells. As for the yield of fluorescence, the direct fluorescence with the phalloidin derivative should be comparable to double immunofluorescence methods because the chance to obtain complexes with actin protomers is much greater for the small toxin molecule ( $M_r$  1250) than for the big immunoglobulin ( $M_r \approx 160,000$ ). It is important to note that phalloidins bind only to the F form of actin.

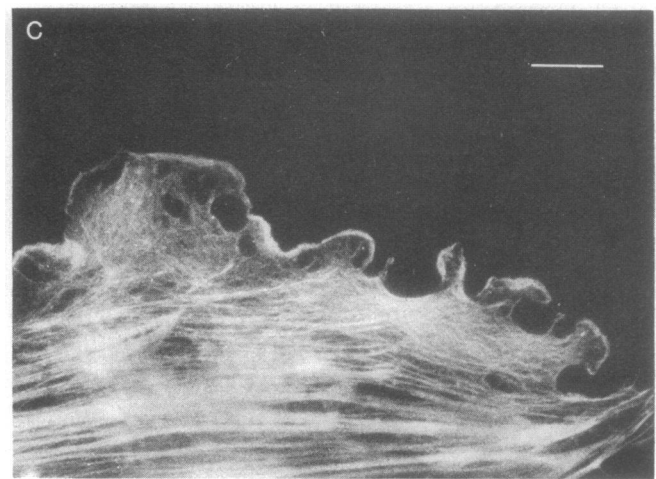
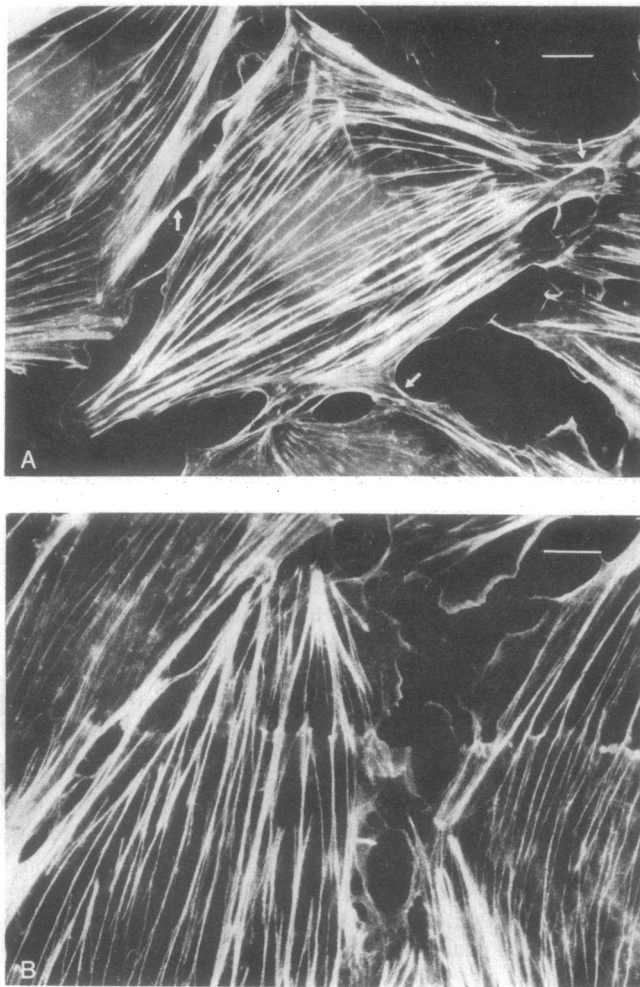


FIG. 1. Direct fluorescent staining of PtK1 cells with FL-phalloidin. (A) Cells stained directly after formaldehyde fixation; arrows indicate junctions of joint fibers. (B and C) Cells stained after formaldehyde fixation followed by Triton X-100 treatment. Note the gauze-like structures at the cell periphery in C. Bars = 10  $\mu$ m.

The fluorescent phalloidin gave bright staining of actin structures in both cell lines investigated. In the flat interphase PtK1 cells we predominantly observed parallel fibers (Fig. 1A) extending the length of the cell and situated submembranously on the adhesive side of the cell, as originally described by Lazarides and Weber (4). Furthermore, in PtK1 cells the actin distribution becomes diffuse when the cells round up for mitosis (Fig. 2). During the phase of mitosis arrangements of actin areas

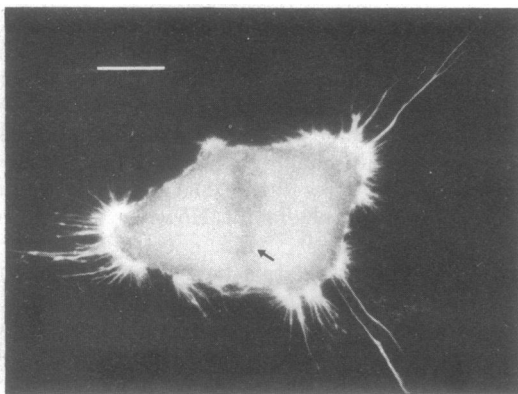


FIG. 2. Mitotic PtK1 cell treated with FL-phalloidin after formaldehyde/acetone fixation. Arrow points to the chromosomes in the metaphase plate. Bar = 10  $\mu$ m.

appear with bright fluorescence located at the periphery of the dividing cell (6, 18). Originating from such actin-rich areas, long projections extend out. These are similar in shape to nerve axons and are also rich in actin.

In the more spherical MDBK cells, quite different actin structures predominate. Most of the actin staining was visible around the nucleus, as a web of thin concentric fibers with radial spokes (Fig. 3A), or as a polygonal array (Fig. 3B) located in more distal regions of the cells (21, 22, 26). In cells having sinus-shaped borders, the concentric web appears to be gathered and stretched into bright fluorescent bundles along the sinus borders. Another type of actin accumulation in MDBK cells was seen in the pseudoruffles (Fig. 3C) (11, 15). Whenever MDBK cells were seen to possess an elongated shape, bundled filaments were also observed (not shown). This agrees with the observation of Willingham *et al.* (27) that microfilament organization of a cell predominantly depends on cell morphology and therefore may change with substrate adhesiveness. By the investigation of several cell lines Willingham *et al.* came to the general conclusion that bundles of filaments occur in flat cells, whereas they are absent in round cells. There is good evidence that phalloidin solely binds to actin. Therefore, the complete disappearance of any decoration pattern when the cells are treated with unlabeled phalloidin proves the high specificity of the new staining reagent.

In conclusion, the fluorescent phalloidin is an excellent tool for studying actin structures in eukaryotic cells. As in the previous investigations, which were carried out with high molecular weight probes, the cells had to be fixed with formaldehyde.

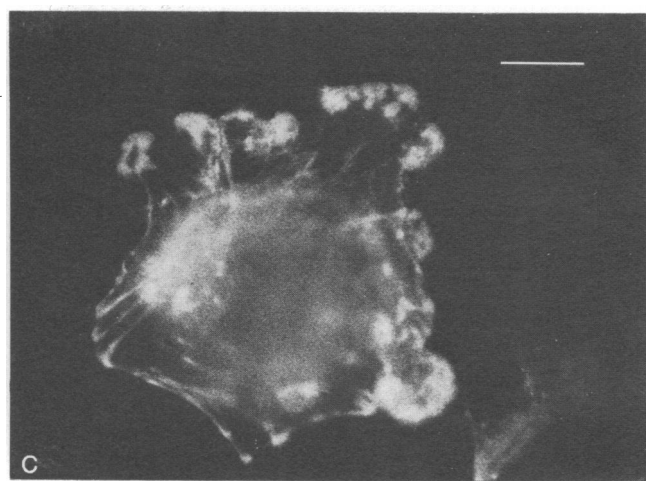
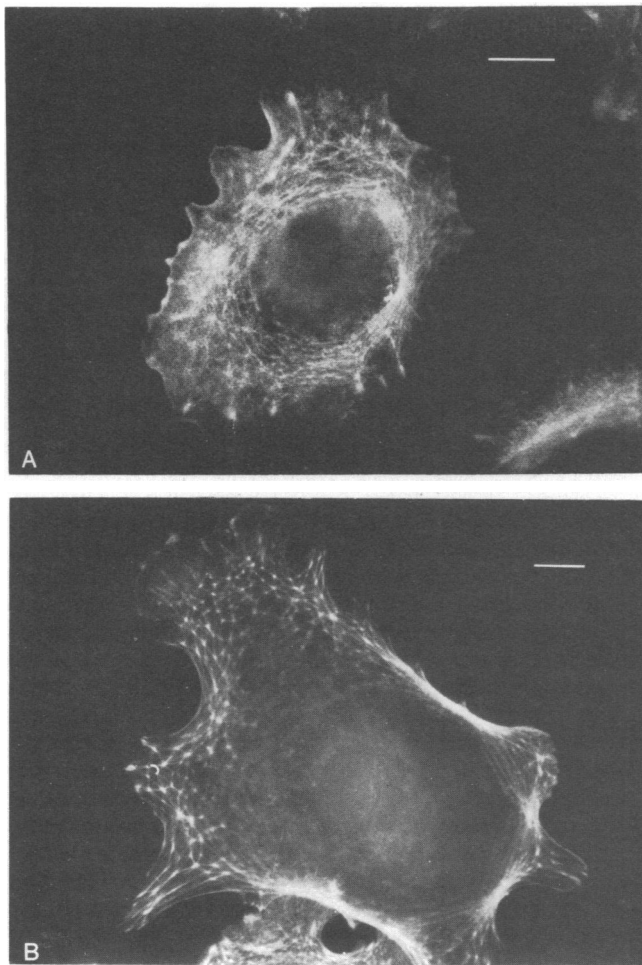


FIG. 3. MDBK cells stained with FL-phalloidin after formaldehyde/acetone (A) or formaldehyde/Triton X-100 treatment (B and C). Note the polygonal arrays of actin foci and connecting fibers (B) and the ruffled membrane (C).

However, in contrast to these, treatment with acetone or detergent prior to staining was no longer obligatory. The low molecular weight of FL-phalloidin should enable living cells to take up the toxin more easily, allowing the distribution of actin to be studied under more stringent *in vivo* conditions.

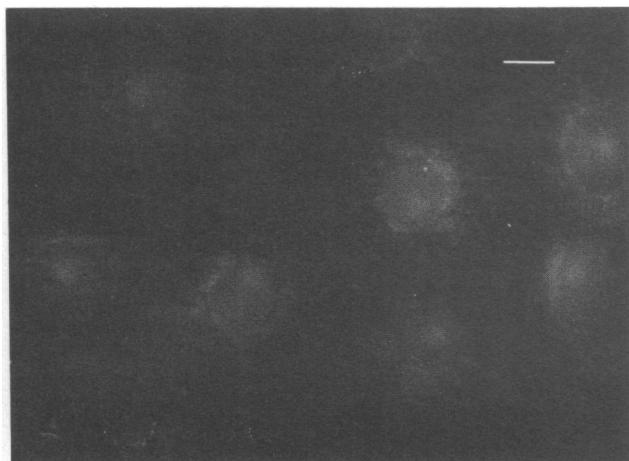


FIG. 4. PtK1 cells pretreated with an excess of unlabeled phalloidin before staining with FL-phalloidin. Bar = 10  $\mu$ m.

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